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Cladosporium hebeiense sp. nov., pathogenic on grape leaves in China

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ABSTRACT—A new fungus in the *Cladosporium herbarum* complex, *Cladosporium hebeiense*, was isolated from leaf spots of *Vitis vinifera* in China. It produced short 0–1-septate ramoconidia that distinguished it from similar species. A multigene phylogenetic analysis based on the internal transcribed spacer region (ITS), partial translation elongation factor 1-alpha (TEF 1-α) gene, and the actin gene (ACT) supported *C. hebeiense* as separate from the closely related *C. ramotenellum*. Pathogenicity testing confirmed that *C. hebeiense* is a pathogen contributing to grape leaf spot disease.

KEY WORDS—*Cladosporiaceae, Capnodiales,* phylogeny, taxonomy,

Introduction

Cladosporium Link is one of the largest and most heterogeneous hyphomycete genera encompassing more than 772 names (Dugan & al. 2004). These fungi are widely distributed throughout the world and have been isolated from plants, soil, food, textile, paint, and other organic materials (Bensch & al. 2012, 2015; Crous & al. 2014). Some *Cladosporium* species are capable of causing human and livestock diseases, and several, including *C. tenuissimum*

Cooke and *C. macrocarpum* Preuss, have been recorded as saprophytes from grapevines in China (Zhang 2003).

At present, several leaf spot diseases of grapevine have been reported in China, caused by *Sultanimyces vitiphyllus* (Speschnew) Videira & Crous (Fan & al. 2007, Song & al. 2014); *Pseudocercospora vitis* (Lév.) Speg. (Chai 2001) and *Passalora dissiliens* (Duby) U. Braun & Crous (Wang & al. 2002, Liang & al. 2010); *Acrospermum viticola* Ikata & Hitomi (Yao 2015); *Septoria ampelina* Berk. & M.A. Curtis, *Grovesinia moricola* (I. Hino) Redhead, and *Pseudopezicula tracheiphila* (Müll.-Thurg.) Korf & W.Y. Zhuang (Wang 2009); and *Neopestalotiopsis vitis* Jayaward. & al. (Jayawardena & al. 2016). Wilcox & al. (2015) have reported several additional grape leaf spot pathogens from other countries, e.g., *Briosia ampelophaga* Cavara, *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud, *Rhytisma vitis* Schwein., *Cladosporium viticola* Ces., *Asperisporium minutulum* (Sacc.) Deighton, and *Passalora heterosporella* U. Braun & Crous.

Here we describe a new *Cladosporium* pathogen, obtained from grape leaf spot in Hebei Province, China. Both morphological and molecular techniques were utilized to evaluate its taxonomic placement.

Materials & methods

Isolates & morphology

Grapevine leaf spot samples were collected from the greenhouse at Changli County, Hebei Province, China in 2016. All the symptomatic leaves were surface-sterilized with 70% ethanol for 1 min, then washed in sterilized water three times, and small pieces (<25 mm^2) of the diseased tissues were placed on potato dextrose agar (PDA) and incubated at 25°C in a 12–12 h light-dark artificial incubator until fungi were observed. Single spore isolations were used to obtain pure cultures as described in Chomnunti & al. (2014). The purified strains were re-cultured on PDA for 14 d at 25°C to observe colony morphologies (Sandoval-Denis & al. 2016). The conidia and conidiophores were photographed using an Axio Imager Z2 photographic microscope, and measurements were taken using ZEN PRO 2012 software. Thirty conidia were measured to calculate size range and average value. Living cultures of the two isolated strains were stored in the culture collection at Beijing Academy of Agricultural and Forestry Sciences, Beijing, China (JZB) and in Guizhou University Culture Collection, Guiyang, China (GUCC); the holotype specimen was conserved as a dried agar culture, in the Herbarium of Beijing Academy of Agricultural and Forestry Sciences (JZBL).

DNA extraction & PCR amplification

Genome DNA was extracted according to Damm & al. (2008). Polymerase chain reactions (PCR) were conducted using C1000 Touch^{TM} thermal Cycler according to

White & al. (1990) with gDNA as the template. The three genes amplified were the internal transcribed spacer region (ITS; using primers ITS4/ITS5, White & al. 1990), partial translation elongation factor 1-alpha gene (TEF 1-α; using primers TEF1-728F/ TEF-2, O'Donnell & al. 1998), and the actin gene (ACT; using primers ACT-512F/ ACT-783R, Bensch & al. 2012). The PCR conditions were as follows—for ITS: initial denaturation for 3 min at 94°C, followed by 34 cycles of denaturation for 30 s at 94°C, 30 s of annealing at 58°C, 90 s elongation at 72°C, and a final extension for 10 min at 72°C; for TEF 1-α and ACT: initial denaturation for 3 min at 94°C, followed by 40 cycles of denaturation for 30 s at 94°C, 30 s of annealing, 90 s elongation at 72°C, and a final extension for 10 min at 72°C. The annealing temperatures used in the cycling program were 54°C for TEF 1-α, and 52°C for ACT. Following PCR amplification, products were visualised on 1% agarose gel stained with ethidium bromide under UV light using a Gel Doc™ XR^+ Molecular Imager. The PCR products were sequenced at Beijing Sunbiotech Co. Ltd.

Phylogenetic analysis

The sequences results were analyzed by BLASTn of National Center for Biotechnology Information (NCBI), and then the relevant reference sequences were downloaded from GenBank (Table 1). The ITS, TEF 1-α, and ACT sequences were aligned using MAFFT v.7 (Katoh & Toh 2008, http://mafft.cbrc.jp/alignment/server/), and manually aligned using BioEdit when necessary (Hall 1999). Then aligned sequences were converted into .nexus format using ALTER alignment online web tool (http://sing.ei.uvigo.es/ALTER/). Maximum parsimony (MP) analysis was conducted using Phylogenetic Analysis Using Parsimony (PAUP) v.4.0b10 (Swofford 2002). The phylogenetic tree was inferred using heuristic search option with tree bisection reconnection (TBR) branch swapping and 1000 random sequence additions, branches of zero length were collapsed and all parsimonious trees were saved. Descriptive statistics included tree length (TL), consistency index (CI), retention index (RI), rescaled consistence index (RC) and homoplasy index (HI). Bootstrap analyses (Hillis & Bull 1993) were based on 1000 replications.

Bayesian analyses were performed in MrBayes v.3.0b4 (Ronquist & Huelsenbeck 2003). The following models were applied: JC+G for the ITS gene region, TrNef+G for the TEF 1-α gene region and TrN+I+G model was selected for ACT gene. The Markov Chain Monte Carlo sampling (MCMC) analysis was conducted with four simultaneous Markov chains. They were run for 1,000,000 generations; sampling the trees at every 100th generation. From the 10,000 trees obtained, the first 2000 representing the burn-in phase were discarded. The remaining 8000 trees were used to calculate posterior probabilities in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01) (Crous & al. 2006). Sequences derived in this study have been deposited in GenBank (Table 1).

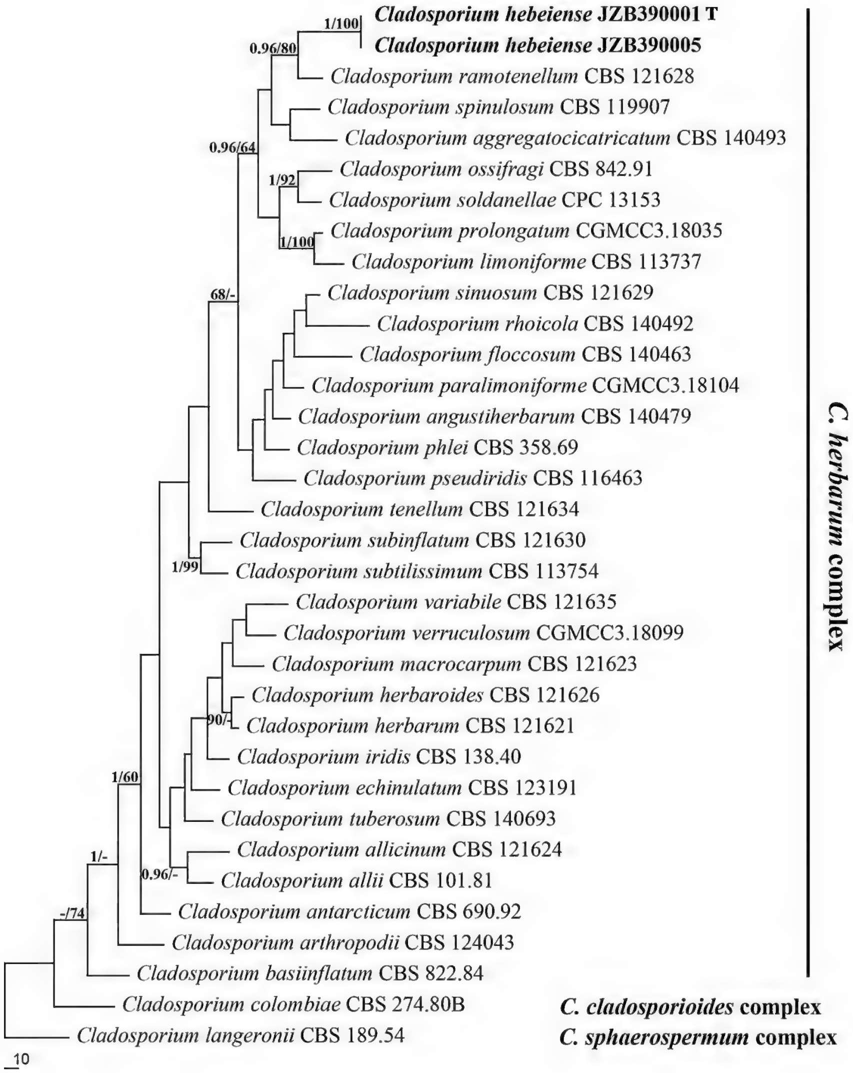
Pathogenicity test

Young and healthy detached *Vitis vinifer*a L. 'Summer Black' leaves collected from the greenhouse were used to test for pathogenicity. Leaves were disinfected with 75%

Table 1. Sequences of *Cladosporium* used in the molecular analyses.

Species	Isolate	GenBank accession numbers			Reference
		ITS	TEF1-α	ACT	
C. aggregatocicatricatum	CBS 140493	KT600448	KT600547	KT600645	Bensch & al. 2015
C. allicinum	CBS 121624	EF679350	EF679425	EF679502	Schubert & al. 2007
C. allii	CBS 101.81	JN906977	JN906983	JN906996	Bensch & al. 2012
C. angustiherbarum	CBS 140479	KT600378	KT600475	KT600574	Bensch & al. 2015
C. antarcticum	CBS 690.92	EF679334	EF679405	EF679484	Schubert &al. 2007
C. arthropodii	CBS 124043	JN906979	JN906985	JN906998	Bensch & al. 2012
C. basiinflatum	CBS 822.84	HM148000	HM148241	HM148487	Bensch &al. 2010
C. echinulatum	CBS 123191	JN906980	JN906987	JN906999	Bensch & al. 2012
C. floccosum	CBS 140463	LN834416	LN834512	LN834600	Sandoval-Denis & al. 2016
C. herbaroides	CBS 121626	EF679357	EF679432	EF679509	Schubert & al. 2009
C. herbarum	CBS 121621	EF679363	EF679440	EF679516	Schubert & al. 2009
C. iridis	CBS 138.40	EF679370	EF679447	EF679523	Schubert & al. 2009
C. limoniforme	CBS 113737	KT600396	KT600493	KT600591	Bensch & al. 2015
C. macrocarpum	CBS 121623	EF679375	EF679453	EF679529	Schubert & al. 2009
C. ossifragi	CBS 842.91	EF679381	EF679459	EF679535	Schubert & al. 2009
C. paralimoniforme	CGMCC3.18103	KX938393	KX938409.	KX938376	Ma & al. 2017

C. phlei	CBS 358.69	JN906981	JN906991	JN907000	Bensch & al. 2012
C. prolongatum	CGMCC3.18035	KX938395	KX938411	KX938378	Ma & al. 2017
C. pseudiridis	CBS 116463	EF679383	EF679461	EF679537	Schubert & al. 2009
C. ramotenellum	CBS 121628	EF679384	EF679462	EF679538	Schubert & al. 2007
C. rhoicola	CBS 140492	KT600440	KT600539	KT600637	Bensch & al. 2015
C. sinuosum	CBS 121629	EF679386	EF679464	EF679540	Schubert & al. 2007
C. soldanellae	CPC 13153	JN906982	JN906994	JN907001	Bensch & al. 2012
C. spinulosum	CBS 119907	EF679388	EF679466	EF679542	Schubert & al. 2007
C. subinflatum	CBS 121630	EF679389	EF679467	EF679543	Schubert & al. 2007
C. subtilissimum	CBS 113754	EF679397	EF679475	EF679551	Schubert & al. 2007
C. tenellum	CBS 121634	EF679401	EF679479	EF679555	Schubert & al. 2007
C. tuberosum	CBS 140693	LN834417	LN834513	LN834601	Sandoval-Denis & al. 2016
C. variabile	CBS 121635	EF679402	EF679480	EF679556	Schubert & al. 2007
C. verruculosum	CGMCC3.18099	KX938389	KX938405	KX938372	Ma & al. 2017
C. hebeiense	**JZB390001 [T]**	**MG516597**	**MG516595**	**MG516593**	**This paper**
	JZB390005	**MG516598**	**MG516596**	**MG516594**	**This paper**
C. colombiae	CBS 274.80B	FJ936159	FJ936163	FJ936166	Schubert & al. 2009
C. langeronii	CBS 189.54	DQ780379	JN906990	EF101357	Zalar & al. 2007


1/100
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Cladosporium hebeiense JZB390005
0.96/80
Cladosporium ramotenellum CBS 121628
Cladosporium spinulosum CBS 119907
Cladosporium aggregatocicatricatum CBS 140493
0.96/64
1/92
Cladosporium ossifragi CBS 842.91
Cladosporium soldanellae CPC 13153
Cladosporium prolongatum CGMCC3.18035
1/100
Cladosporium limoniforme CBS 113737
68/-
Cladosporium sinuosum CBS 121629
Cladosporium rhoicola CBS 140492
Cladosporium floccosum CBS 140463
Cladosporium paralimoniforme CGMCC3.18104
Cladosporium angustiherbarum CBS 140479
Cladosporium phlei CBS 358.69
Cladosporium pseudiridis CBS 116463
Cladosporium tenellum CBS 121634
Cladosporium subinflatum CBS 121630
1/99
Cladosporium subtilissimum CBS 113754
Cladosporium variabile CBS 121635
Cladosporium verruculosum CGMCC3.18099
Cladosporium macrocarpum CBS 121623
Cladosporium herbaroides CBS 121626
90/-
Cladosporium herbarum CBS 121621
Cladosporium iridis CBS 138.40
1/60
Cladosporium echinulatum CBS 123191
Cladosporium tuberosum CBS 140693
1/-
Cladosporium allicinum CBS 121624
0.96/-
Cladosporium allii CBS 101.81
-/74
Cladosporium antarcticum CBS 690.92
Cladosporium arthropodii CBS 124043
Cladosporium basiinflatum CBS 822.84
Cladosporium colombiae CBS 274.80B
Cladosporium langeronii CBS 189.54
10
C. herbarum complex
C. cladosporioides complex
C. sphaerospermum complex

Fig. 1. Phylogram based on maximum parsimony and Bayesian analyses with combined ITS, TEF1-α, and ACT sequence data of *Cladosporium* species. Bootstrap values >50% and posterior probabilities >0.96 are indicated near the nodes. Sequences from our new species are set in bold font. The scale bar indicates 10 changes. The tree is rooted with *C. langeronii* [*C. sphaerospermum* complex] and *C. colombiae* [*C. cladosporioides* complex].

alcohol for 30 s, washed with sterile water 3 times, and then placed in sterile operating platform for drying. We re-cultured the isolates on PDA for 7 d, prepared a 1 × 10^5/ml spore suspension, and conducted the pathogenicity test using three leaves per isolate with the drip method. Leaves were inoculated using two inoculation methods: non-wounded and wounded, with 4 inoculation points on each leaf (2 inoculation points on each side) with a spore suspension of 20 µl. Inoculated leaves were placed into an inoculation box for moisturizing, and the box was placed in an incubator at 25°C for 12 h light/12 h dark. The pathogenic experiment was repeated 3 times, with sterile water serving as the negative control. After the onset of disease symptoms, the fungus was re-isolated, fulfilling Koch's postulates.

Phylogenetic results

The phylogenetic tree constructed by MP comprised 30 sequences from the *Cladosporium herbarum* complex (Bensch & al. 2012, 2015), with *Cladosporium langeronii* (CBS 189.54; *C. sphaerospermum* complex) and *C. colombiae* (CBS 274.80B; *C. cladosporioides* complex) as the outgroup. After the three gene regions (ITS, TEF 1-α, and ACT) were combined, the final alignment was 1048 characters long (ITS: 1–478, TEF 1-α: 479–859, ACT: 860–1048). The concatenated alignment contained 229 parsimony informative characters, 707 constant characters, and 112 variable and parsimony uninformative characters. One (TL = 1259, CI = 0.448, RI = 0.529, RC = 0.237, HI = 0.552) of 1000 equally parsimonious trees is shown in Fig. 1. Within the *Cladosporium herbarum* complex, our two isolates (JZB390001, JZB390005) clustered together with maximum parsimony bootstrap (MP) = 100% and Bayesian posterior probability (PP) = 1. Our isolates clustered with their sister taxon, *C. ramotenellum* K. Schub. & al. with MP = 80% and PP = 0.96.

Taxonomy

Cladosporium hebeiense C.X. Wang, Xing H. Li & Yan M. Wei, **sp. nov.** Fig. 2

Index Fungorum IF553970

Differs from *Cladosporium ramotenellum* by its shorter, wider ramoconidia with fewer septa.

Type: China, Hebei Province, Changli County, on leaf spot of greenhouse *Vitis vinifera* (*Vitaceae*), November 2016, Xinghong Li (Holotype, JZBL390001; ex-holotype cultures, JZB390001, GUCC3075; GenBank MG516597, MG516595, MG516593).

Etymology: Referring to the collection locality in Hebei Province.

Colonies: On PDA 45–55 mm diam after 14 d at 25°C, moderate green to dark green, flat to umbonate and slightly folded, velvety with white cottony centre and regular margin; reverse olive brown. On OA 43–47 mm diam after

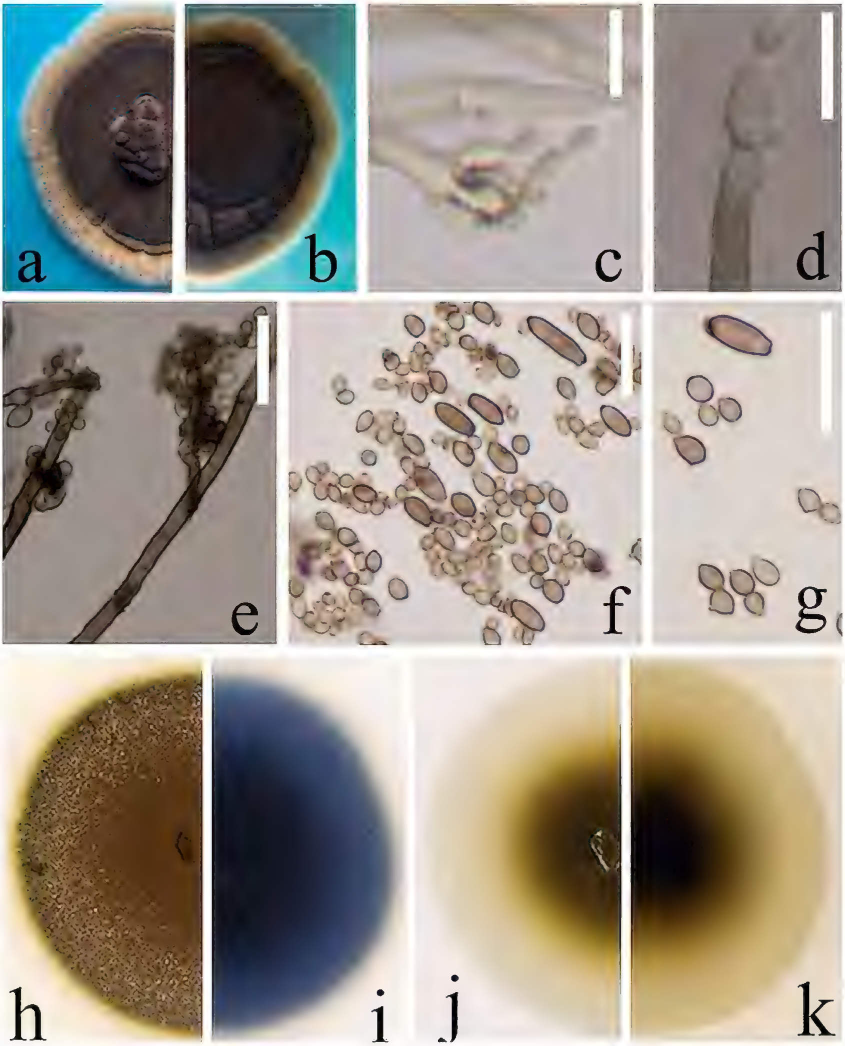

Fig. 2. *Cladosporium hebeiense* (JZB3900005, ex-holotype). a, b. Colonies on PDA (a-obverse, b-reverse) at 25°C; c, d. Sporulation with primary conidia on PDA; e. Conidiophores and conidia on PDA; f, g. Two kinds of conidia on PDA; h, i. Colonies on OA (a = obverse, b = reverse) at 25°C; j, k. Colonies on SNA (a = obverse, b = reverse) at 25°C. Scale bars: c, e, f = 5 µm; d, g = 10 µm.

14 d at 25°C, olive green or olive brown, nearly round, powdery, reverse dark olive green to black. On SNA 32–34 mm diam after 14 d at 25°C, yellowish-brown to dark brown, aerial hyphae not obvious, nearly round, reverse back

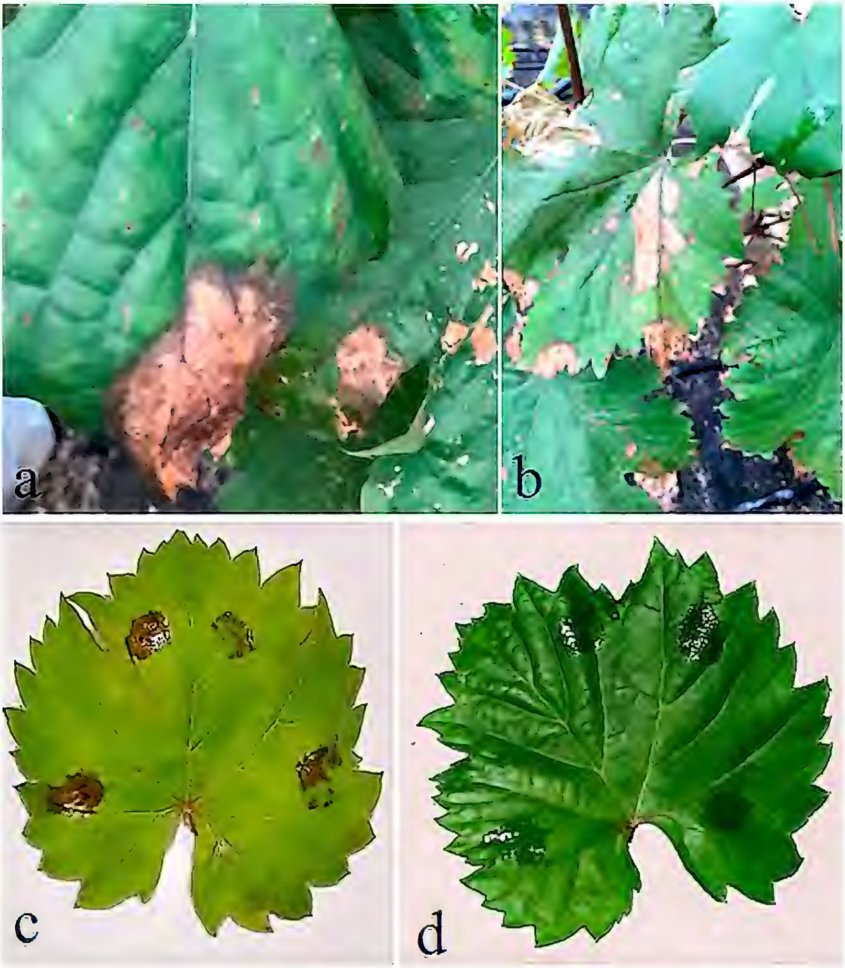

Fig. 3. Symptoms of grape leaf spot disease associated with *Cladosporium hebeiense*. a, b. Field symptoms of grape leaf spot; c. Symptoms on in vitro grape leaves, 7 d after inoculation with isolate JZB3900001. d. In vitro grape leaves, 7 d after control inoculation with sterile water.

brown to dark brown. Mycelium superficial and immersed, hyphae septate, branched, 1.5–3 µm wide, sub-hyaline to pale brown, smooth. Conidiophores erect, flexuous, sub-cylindrical, septate, mostly unbranched, ≤150 × 3–3.5 µm, pale to medium olivaceous brown, smooth. Conidiogenous cells terminal, cylindrical, nodulose, 18–20 × 3–4.5 µm, smooth and thick-walled, bearing up to 2–3 conspicuous, refractive, slightly darkened conidiogenous loci of 2–2.5

µm diam. Ramoconidia 0–1-septate, subcylindrical to cylindrical, 7.5–20 × 4–5.5 µm (mean = 10.24 × 4.54 µm, n = 30), smooth or finely verruculose. Conidia forming branched chains with ≤5–7 conidia at the unbranched terminus, pale green-brown, smooth- and thick-walled, with protuberant and darkened conidial hila; small terminal conidia aseptate, obovoidal to short ellipsoidal, 3–8 × 3–5 µm (mean = 5.48 × 3.88 µm, n = 30).

Additional material examined: **CHINA, Hebei Province, Changli County**, on leaf spot of greenhouse *Vitis vinifera*, November 2016, Xinghong Li (cultures (JZB390005, GUCC3076; GenBank MG516598, MG516596, MG516594).

Pathogenicity

This grape leaf spot is primarily characterized as light brown spots with a darker edge, circular, oval, or irregular in shape, mainly concentrated in the relatively old leaves (Fig. 3). The leaves begin to show symptoms at 3 d. Symptoms were similar to those of field acquired leaf spot disease at 7 d. No symptoms were observed on the control (Fig. 3). After Koch's postulates, re-isolated cultures, and morphological observation of ramoconidia and conidia confirmed the pathogen was *Cladosporium hebeiense*.

Discussion

As can be seen from the phylogenetic tree (Fig. 1), *Cladosporium hebeiense* falls within the *C. herbarum* complex and is closely related to *C. ramotenellum* with strong support (MP = 80%, PP = 0.96). However, *C. ramotenellum* differs from *C. hebeiense* by its longer, narrower ramoconidia with more numerous septa (<47 × 2–4 µm, 0–1(–4)-septate; Bensch & al. 2012, Schubert & al. 2007). Based on morphological comparison and phylogenetic analysis, we confirm that *C. hebeiense* is a new taxon in the *C. herbarum* complex.

Cladosporium species are very common fungi that cause leaf spot diseases in many plants such as rock lily, wheat, *Vigna umbellata, Citrus sinensis,* and *Colocasia esculenta* (Bensch & al. 2012, Sandoval-Denis & al. 2016). However, at present there are no other reports from China of grape leaf spots caused by *Cladosporium*. Our report not only adds a new *Cladosporium* species, but also promotes the research progress of grape leaf spot in China. Our next step will be to investigate the main grape producing areas in China to evaluate the impact of *C. hebeiense* on Chinese grapes.

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